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Exposure to Fine Particulate Air Pollution Causes Vascular Insulin Resistance by Inducing Pulmonary Oxidative Stress

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Running title: $PM_{2.5}$ disrupts vascular insulin signaling

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ABSTRACT

Background: Epidemiological evidence suggests that exposure to ambient air fine particular matter ($PM_{2.5}$) increases the risk of developing type 2 diabetes and cardiovascular disease. However, the mechanisms underlying these effects of $PM_{2.5}$ remain unclear.

Objectives: We tested the hypothesis that $PM_{2.5}$ exposure decreases vascular insulin sensitivity by inducing pulmonary oxidative stress.

Methods: Mice fed control (10-13% kcal fat) and high-fat (60% kcal fat, HFD) diet, treated with 4-hydroxy-2,2,6,6-tetramethylpiperidine1-oxyl (TEMPOL) or mice overexpressing lung-specific extracellular superoxide dismutase (ecSOD) were exposed to HEPA-filtered air or concentrated $PM_{2.5}$ (CAP) for 9 or 30 days and changes in systemic and organ-specific insulin sensitivity and inflammation were measured.

Results: In control diet-fed mice, CAP exposure for 30 days decreased insulin-stimulated Akt phosphorylation in lung, heart, and aorta but not in skeletal muscle, adipose tissue and liver and did not affect adiposity or systemic glucose tolerance. In HFD-fed mice, 30-day CAP exposure suppressed insulin-stimulated endothelial nitric oxide synthase (eNOS) phosphorylation in skeletal muscle, and increased adipose tissue inflammation and systemic glucose intolerance. In control diet-fed mice, a 9-day CAP exposure was sufficient to suppress insulin-stimulated Akt and eNOS phosphorylation and to decrease IκBα levels in the aorta. Treatment with the antioxidant TEMPOL or lung-specific overexpression of ecSOD prevented CAP-induced vascular insulin resistance and inflammation.

Conclusions: Short-term exposure to $PM_{2.5}$ induces vascular insulin resistance and inflammation triggered by a mechanism involving pulmonary oxidative stress. Suppression of vascular insulin signaling by $PM_{2.5}$ may accelerate the progression to systemic insulin resistance, especially in the context of diet-induced obesity.

INTRODUCTION

Recent studies suggest that urbanization and the associated increase in air pollution could be a contributing factor to the world-wide increase in the incidence of diabetes (Bhatnagar 2009; Rao et al. 2015). Because the rates of obesity and type 2 diabetes (T2D) have increased significantly in only a few generations (CDC 2012), it is likely that this increase is attributable, at least in part, to environmental factors such as lifestyle choices, community environment and exposure to polluted air, rather than to population-wide genetic changes. In agreement with this view, several epidemiological studies have shown that exposure to air pollution increases the risk of T2D (Chen et al. 2013; Coogan et al. 2012; Eze et al. 2015; Kramer et al. 2010; Park et al. 2015; Pearson et al. 2010). Exposure to ambient air pollution has also been linked to an increase in diabetes-associated mortality (Brook et al. 2013a; Pope et al. 2015), exacerbation of cardiometabolic disorders (Pope et al. 2015), and poor metabolic control in individuals with (Tamayo et al. 2014) or without diabetes (Brook et al. 2013b). An analysis of individual US counties found a significant positive association between ambient levels of air fine particulate matter (≤2.5μm, $PM_{2.5}$) and the prevalence of T2D, but not obesity (Pearson et al. 2010), suggesting that exposure to air pollution may be an obesity-independent T2D risk factor. Taken together, these studies raise the possibility that the current epidemic of T2D may be due, in part, to recurrent exposure to high levels of ambient air pollutants.

Experimental data from animal studies support the idea that there is a biologically plausible link between $PM_{2.5}$ exposure and the development of diabetes. Specifically, it has been reported that in mice fed a high-fat diet (HFD), prolonged exposure to concentrated ambient $PM_{2.5}$ increases systemic insulin resistance and visceral adiposity (Sun et al. 2009). Long-term exposure for 10 months has been found to induce systemic insulin resistance in mice, even in the

absence of a HFD (Xu et al. 2011). Although these studies found that $PM_{2.5}$ induces metabolic dysfunction, it remains unclear how this defect develops and whether the systemic effects of $PM_{2.5}$ are linked to and are preceded by changes specific to cardiovascular tissues. This is important because $PM_{2.5}$ exposure has been associated with an increase in cardiovascular disease (CVD) risk (Brook et al. 2010), which may be related to defects in insulin signaling specifically in cardiovascular tissues, independent of systemic insulin resistance.

In models of diet-induced obesity, vascular insulin resistance precedes the development of insulin resistance in skeletal muscle, liver, and adipose tissue (Kim et al. 2008), suggesting that the development of "early" vascular insulin resistance plays a critical, if not an essential, role in the subsequent development of systemic glucose intolerance. While vascular dysfunction is an early event in the development of diabetes (Kim et al. 2006), defective insulin signaling in the vasculature has been reported to be sufficient and necessary for the development of systemic insulin resistance (Kubota et al. 2011). Nevertheless, it remains unclear how inhaled $PM_{2.5}$ affects insulin signaling in blood vessels. Hence, we examined the effects of $PM_{2.5}$ exposure to determine whether changes in vascular insulin signaling are secondary to diet-induced changes and an increase in systemic or pulmonary oxidative stress, because oxidative stress plays a well described role in mediating the toxicity of $PM_{2.5}$ and mice deficient in the NADPH oxidase subunit p47(phox), which generates reactive oxygen species (ROS), are protected against the effects of $PM_{2.5}$ exposure on systemic insulin resistance (Xu et al. 2010).

We found that short-term inhalation of concentrated ambient $PM_{2.5}$ (CAP) induces vascular insulin resistance independent of dyslipidemia, obesity and systemic inflammation and that $PM_{2.5}$–induced vascular insulin resistance could be mitigated by increasing the removal of superoxide specifically in the lung. These findings reveal a novel link between pulmonary

oxidative stress and vascular insulin resistance - a link that may explains how air pollution increases the risk of developing both cardiovascular and metabolic disease.

METHODS

Animal studies. Mice were treated humanely according to the APS's Guiding Principles in the Care and Use of Animals following protocols approved by the University of Louisville IACUC. Male, ≈12-weeks old C57BL/6 mice fed a control (10-13% kcal fat) or a high-fat diet (HFD, 60% kcal fat, Fig. 1-3, Studies I-III), treated with 4-hydroxy-2,2,6,6-tetramethylpiperidine1-oxyl (TEMPOL, Fig. 4, Study IV) and mice transgenic (Tg) for lung-specific extracellular superoxide dismutase (ecSOD, Fig. 5, Study V) were exposed to HEPA-filtered air or concentrated ambient $PM_{2.5}$ (CAP, 6 h/d) for 9 or 30 consecutive days as described in the Supplemental Material, Methods. Body weight was measured weekly and a glucose tolerance test (GTT) was performed (day 25, Study I). For GTT, mice fasted for 6h were injected with glucose (1 g/kg bwt, i.p.) and blood glucose levels were monitored (ACCU-CHECK, Aviva; Roche). Finally, after 6h food withdrawal, blood and organs were collected. Plasma was used for biochemical analysis as described (Conklin et al. 2009) or using commercial kits (Mouse Multi/Singleplex-adipokine/adiponectin, Luminex 200, Millipore; TBARS, ZeptoMetrix). Insulin signaling was examined in organs of mice injected (i.p., 0.1 ml, 15 min, Study II) with saline or insulin (Humulin-RP, Eli-Lilly, 1.5 U/kg), or in isolated aorta (Studies III-V) (Haberzettl et al. 2012) stimulated *ex vivo* with vehicle or insulin (100 nM, 15 min).

Immunoblotting. Western blot analyses were performed using indicated antibodies as described in the Supplemental Material, Methods.

Statistical analyses. Data are mean ± SE. Statistical significance ($P < 0.05$) was determined using an unpaired Student's *t*-test or one-way ANOVA with Bonferroni post-hoc test (SigmaStat, SPSS) where appropriate. To analyze the dose-response of CAP concentration and aortic insulin signaling, a nonlinear first order exponential equation [$(y=y_0 + a_1 \exp(-x/t_1)$], chosen based on the regression coefficient R and χ^2 test, was fitted to the data.

RESULTS

Effects of CAP exposure on systemic glucose homeostasis. To examine how $PM_{2.5}$ exposure affects systemic insulin resistance, we exposed control and HFD-fed mice for 30 days to air or CAP (Study I, Fig. 1) and measured systemic glucose tolerance by GTT and calculated HOMA-IR and HOMA-β scores, which reflect systemic insulin resistance and insulin release, respectively. We found that in control diet fed mice, CAP exposure neither led to systemic glucose intolerance (Fig. 1A) nor changes in body weight, fasting blood glucose, plasma insulin levels, and the HOMA-IR and HOMA-β scores (Table 1), indicating that the mice were not insulin resistant or glucose intolerant. CAP-exposed mice, however had higher plasma levels of total and LDL cholesterol and a lower HDL:LDL ratio than air-exposed mice (Table 1). CAP exposure did not affect the plasma levels of major adipokines (adiponectin, leptin, resistin), cytokines (TNF-α, IL-6) or markers of liver (total protein; albumin; alanine aminotransferase, ALT; aspartate aminotransferase, AST) and muscle (creatinine kinase, CK) injury (Table 1). Moreover, there was no increase in pulmonary inflammation as assessed by measuring the levels of IL-1β, IL-6, MCP-1 and TNF-α mRNAs in the lungs (see Supplemental Material, Table S3). Collectively, these data indicate that CAP exposure for 30 days induces mild dyslipidemia, but not systemic insulin resistance, obesity, lung inflammation or overt muscle or hepatic injury.

The absence of CAP-induced changes in glucose tolerance and HOMA-IR scores suggests that PM-exposure does not induce systemic insulin resistance in mice fed control diet; however, to test whether $PM_{2.5}$ exposure exacerbates pre-existing insulin resistance, we examined the effects of CAP exposure in mice with insulin resistance induced by HFD (Study I; Fig. 1). We found that CAP exposure significantly exacerbated both glucose intolerance (Fig. 1B, see increased GTT AUC, inset) and HOMA-IR score in these mice (Table 1), however, CAP exposure did not affect HOMA-β score, suggesting that pancreatic β-cell function was unaltered (Table 1). Exposure to CAP also increased the plasma levels of total cholesterol, although no change in the levels of adipokines, cytokines or muscle or liver enzymes was observed in comparison with air-exposed, HFD-fed mice (Table 1). CAP exposure did not alter body weight gain or adiposity, but it did attenuate HFD-induced adipocyte hypertrophy and it enhanced adipose tissue inflammation as reflected by increased levels of $F4/80^{+}$-cells, crown-like structures (CLS) and of TNF-α and MIP-1α mRNA levels in the adipose tissue (see Supplemental Material, Fig. S1A-E). Nevertheless, no changes in pro-inflammatory mRNA levels in the lungs were apparent (see Supplemental Material, Table S3). These observations suggest that CAP exposure exacerbates HFD-induced systemic insulin resistance and adipose tissue inflammation, independent of changes in adiposity, pulmonary inflammation and systemic toxicity.

Effects of CAP exposure on organ-specific insulin sensitivity. Previous work has shown that in rodent models of diet-induced obesity, vascular insulin resistance (measured as decreased insulin-stimulated Akt and eNOS phosphorylation) is necessary and sufficient for, and thus precedes, the subsequent development of organ-specific and systemic insulin resistance (Kim et al. 2008; Kubota et al. 2011). Nevertheless, it is unknown whether CAP exposure induces vascular insulin resistance and whether this precedes organ-specific insulin resistance.

Hence, to examine the effect of CAP exposure on changes in organ-specific insulin sensitivity, we exposed control and HFD-fed mice to air or CAP for 30 days (Study II, Fig. 2). In control diet-fed mice, CAP exposure diminished insulin-stimulated Akt phosphorylation in the lungs (Fig. 2A), heart (Fig. 2B), and aorta (Fig. 2Ci), as well as eNOS phosphorylation in the aorta (Fig. 2Cii) without affecting insulin signaling in the skeletal muscle (Fig. 2D) or decreasing the abundance of the aortic or cardiac insulin receptor (data not shown). These results indicate that CAP exposure causes early decreases in insulin-sensitivity in the lung, heart and aorta, which precede the development of systemic insulin resistance (Fig. 1A).

As expected, feeding HFD decreased insulin-stimulated Akt phosphorylation in the heart, aorta and skeletal muscle but this defect was not further exacerbated by CAP exposure (Fig. 2). In HFD-fed mice, CAP exposure did not further suppress insulin-stimulated eNOS phosphorylation in the aorta (Fig. 2Cii) but it completely prevented insulin-stimulated eNOS phosphorylation in the skeletal muscle (Fig. 2Dii). This decrease in total skeletal muscle eNOS phosphorylation (Fig. 2Cii) was associated with an increase in whole-body insulin resistance (Fig. 1B), suggesting that the CAP-induced increase in systemic glucose intolerance in HFD-fed mice might be related to an increase in endothelial insulin resistance in the skeletal muscle. CAP exposure decreased eNOS phosphorylation (likely an endothelial-specific event) without affecting Akt phosphorylation (Akt is ubiquitous); suggesting that CAP exposure specifically affects the endothelium. Moreover, because CAP exposure did not affect insulin-stimulated Akt phosphorylation in the adipose tissue or the liver (data not shown), it appears that disruption of insulin signaling in the endothelium of the skeletal muscle is sufficient to account for the increase in systemic glucose intolerance in CAP-exposed mice.

CAP exposure induces early vascular insulin resistance and inflammation. Because the results of Studies I and II show that CAP exposure for 30 days impairs insulin signaling in multiple tissues, we asked whether a shorter duration of exposure would induce cardiovascular insulin resistance. For this, we exposed control and HFD-fed mice to air or CAP for 9 days (Fig. 3; Study III) and then measured insulin signaling in the aorta and heart *ex vivo* (to preclude systemic effects of insulin such as changes in blood flow and blood pressure). We found that a 9-day CAP exposure suppressed insulin-stimulated Akt phosphorylation in the heart (see Supplemental Material, Fig. S2A). In the aorta, 9-day CAP also exposure decreased insulin-stimulated phosphorylation of Akt (Fig. 3Ai) and eNOS (Fig. 3Aii) but not ERK (Fig. 3Aiii, see Supplemental Material, Fig. S2B). These changes were accompanied by a decrease in aortic IκBα levels (Fig. 3Aiv). Collectively, these results show that CAP exposure activates the pro-inflammatory NFκBα-pathway and selectively impairs the insulin-induced activation of the PI3K/Akt/eNOS-pathway but not the MAPK/ERK-pathway.

Having found that a decrease in insulin-stimulated Akt phosphorylation in the aorta is a sensitive measure of CAP-induced injury; we examined the dose-dependence of this response. As shown in Fig. 3B, we found a nonlinear relationship between CAP concentration and vascular insulin resistance with an ED_{50} of 82 $\mu g/m^3$. This dose corresponds to a $PM_{2.5}$ exposure level of $\approx$ 20 $\mu g/m^3$ for 24h, which is comparable to the CAP concentrations used in previous animal studies (Sun et al. 2009; Xu et al. 2011) and similar to the 24h average levels of $PM_{2.5}$ in most major US cities, which vary from 20-35 $\mu g/m^3$ (Brook et al. 2010).

Because our results showed that CAP exposure attenuates aortic insulin signaling, we next examined whether this was accompanied by vascular dysfunction. We found that CAP exposure (for 9 or 30 days) did not induce frank endothelium dysfunction measured as

acetylcholine-mediated relaxation in the aorta (Study III and I; data not shown); however, CAP exposure for 9 days potentiated the contractile responses of the aorta to both phenylephrine (alpha-adrenergic agonist) and high potassium (non-receptor agonist) (see Supplemental Material, Fig. S2Ci and Table S5), which may also reflect a diminished endothelium-dependent relaxation capacity (Walker et al. 1997). When the exposure duration was extended to 30 days, the early hypercontractile response switched to hypocontractility (see Supplemental Material, Fig. S2Cii and Table S5) - a condition already present in the aorta of HFD-fed mice (see Supplemental Material, Fig. S2Ci and ii and Table S5). These data indicate that even in the absence of HFD, short-term CAP exposure results in early vascular dysfunction.

Role of systemic oxidative stress in mediating CAP-induced vascular insulin resistance and inflammation. To examine the mechanism of CAP-induced injury, we charted early systemic changes that could contribute to the development of vascular insulin resistance. For this, we measured plasma biomarkers of inflammation and metabolic injury. Although we found that a 9-day CAP exposure did not affect blood glucose, plasma insulin levels, HOMA-IR, HOMA-β scores, or plasma lipids and proteins (Table 2), this short-term exposure did increase the abundance of plasma acrolein-protein adducts (Fig. 3C) and plasma TBARS level (Fig. 3C, inset), which are reflective of increased systemic oxidative stress and lipid peroxidation (Uchida et al. 1998). These observations suggest that CAP exposure increases systemic oxidative stress and lipid peroxidation - changes that accompany vascular insulin resistance and inflammation (Fig. 3A) and precede the development of overt systemic inflammation, systemic insulin resistance and dyslipidemia (Table 2).

To determine whether this early oxidative stress was causally related to vascular insulin resistance and inflammation, we treated mice with the antioxidant TEMPOL and exposed them

to air or CAP (Fig. 4, Study IV). Consistent with our previous results, we found that CAP exposure attenuated insulin-stimulated Akt phosphorylation and decreased IκBα levels in the aorta; however, TEMPOL treatment prevented both changes (Fig. 4A-B). These results support the notion that CAP exposure induces oxidative stress that, in turn, suppresses vascular insulin signaling and induces vascular inflammation.

Role of pulmonary oxidative stress in mediating CAP-induced vascular insulin resistance and inflammation. Given that the lungs are the first and major site of inhaled particle deposition and toxicity (Oberdorster et al. 2005), we measured oxidative stress in the lungs of CAP-exposed mice to determine whether CAP exposure induces pulmonary oxidative stress. For this, we measured changes in pulmonary antioxidant genes, because an increase in these genes would be reflective of pulmonary oxidative stress. We found that 9-day CAP exposure caused an increase in the expression of antioxidant genes - SOD2, SOD3 and GST-α - (see Supplemental Material, Table S6) and correspondingly an increase in the protein abundance of ecSOD (SOD3, extracellular superoxide dismutase, Fig. 5A) in the lungs, which could be indicative of pulmonary oxidative stress. However, indices of pulmonary inflammation (see Supplemental Material, Table S3**)** were unaffected, suggesting that CAP exposure results in acute pulmonary oxidative stress without triggering overt lung inflammation.

The increase in the levels of antioxidant enzymes in the lung of CAP exposed mice, led us to ask whether oxidative stress in the lungs was related to the development of vascular insulin resistance and inflammation in CAP-exposed mice. Therefore, we tested whether increasing the antioxidant capacity of the lung would diminish these vascular effects of CAP. To enhance the antioxidant capacity of the lung, we exposed mice transgenic for lung-specific ecSOD (ecSOD-Tg) (Folz et al. 1999), which show lung-restricted overexpression of the transgenic ecSOD gene

(Fig. 5B), to air or CAP for 9 days (Study V) and examined changes in protein-acrolein adducts in the lungs. As shown in Fig. 5C**,** CAP exposure increased the levels of protein-acrolein adducts in the lungs of WT (i) but not of ecSOD-Tg mice (ii), indicating that overexpression of ecSOD prevents CAP-induced pulmonary oxidative stress. Remarkably, we found that the overexpression of ecSOD exclusively in the lung also prevented CAP-induced oxidative stress in peripheral tissues such as circulating lymphocytes and the aorta (see Supplemental Material, Fig. S3A-B). Moreover, as shown in Fig. 5D and 5E**,** even though CAP exposure attenuated insulin sensitivity and decreased IκBα levels in the aorta of WT mice, these effects were absent in CAP-exposed ecSOD-Tg mice. Similarly, overexpression of pulmonary ecSOD also attenuated the CAP-induced decrease in plasma NO_x levels (see Supplemental Material, Table S7). Taken together, these results suggest that the overexpression of ecSOD in the lungs prevents the development of vascular insulin resistance and inflammation in CAP-exposed mice, and support the idea that CAP exposure increases pulmonary oxidative stress that, in turn, triggers systemic oxidative stress leading to the development of vascular insulin resistance and inflammation.

DISCUSSION

This study shows that short-term exposure to $PM_{2.5}$ decreases diet-independent vascular insulin sensitivity, which is mediated in part by oxidative stress in the lungs. These findings provide a novel link between pulmonary oxidative stress and vascular insulin signaling by showing that pulmonary oxidative stress is sufficient to induce insulin resistance and inflammation in blood vessels of mice exposed to $PM_{2.5}$. Because insulin resistance localized to the vasculature is a sub-clinical effect, which could occur in the absence of measurable systemic effects, this outcome has not been observed in previous clinical studies. Nevertheless, it might

represent a critical first step of the mechanism by which $PM_{2.5}$ exposure accelerates and exacerbates the risk for both cardiovascular and metabolic diseases in humans.

Previous studies have shown that acute and chronic exposures to ambient levels of $PM_{2.5}$ are associated with an increase in the prevalence for CVD and diabetes as well as enhanced systemic insulin resistance and diabetes-related mortality in humans (Brook et al. 2010; Brook et al. 2013a; Chen et al. 2013; Coogan et al. 2012; Eze et al. 2015; Park et al. 2015; Pearson et al. 2010; Pope et al. 2015; Rao et al. 2015). Nevertheless, the mechanisms underlying these changes remain unclear. The ability of $PM_{2.5}$ to decrease systemic insulin sensitivity has also been demonstrated in animal models, which show that prolonged (6 to 10 months) $PM_{2.5}$ exposure increases systemic insulin resistance (Sun et al. 2009; Xu et al. 2011). However, our observation that short-term $PM_{2.5}$ exposure induces vascular insulin resistance, without affecting systemic insulin sensitivity, suggests that in comparison with other tissues, the blood vessels are more sensitive to $PM_{2.5}$ exposure and that vascular insulin resistance could be a contributing factor to the development of CVD and diabetes in humans exposed to ambient air pollution. In particular, the observed decrease in insulin-stimulated eNOS phosphorylation in CAP-exposed animals suggests that $PM_{2.5}$-induced endothelial insulin resistance could be a key event in the mechanism triggering the onset of other deleterious cardiovascular outcomes and systemic insulin resistance after $PM_{2.5}$ exposure. Phosphorylation of eNOS by insulin increases eNOS activity and NO production, which regulates vascular tone, thrombosis and atherogenesis (Kim et al. 2008; Kim et al. 2006; Landmesser et al. 2004). Therefore, the suppression of insulin-stimulated phosphorylation of eNOS could account for many of the vascular effects of $PM_{2.5}$ such as the increases in blood coagulation, blood pressure, vascular dysfunction, and atherogenesis (Brook et al. 2010). Previous work has shown that cardiovascular deaths account for a majority of premature mortality associated with exposure to particulate air pollution and

these outcomes have been linked to an increase in inflammation as well as PM-induced dysfunction in the endothelium and the autonomic and central nervous system (Brook et al. 2010). Although changes in each of these processes could individually elevate CVD risk; it remains unclear whether $PM_{2.5}$ provokes cardiovascular and metabolic injury by simultaneously affecting all these processes at once or only a core set of "sensitive" processes. Our current data, showing that exposure to $PM_{2.5}$ suppresses vascular insulin signaling, suggests that vascular insulin resistance could be one such "sensitive" target of $PM_{2.5}$ that, in turn affects many different processes such as tissue perfusion, endothelial function, and atherogenesis (Kim et al. 2006; Kubota et al. 2011; Landmesser et al. 2004). Collectively, theses changes could increase cardiometabolic risk and cardiovascular mortality in humans exposed to $PM_{2.5}$. In this regard, it is significant to point out that the effects observed in our study occurred at exposure levels near the National Ambient Air Quality Standard for $PM_{2.5}$ (12 $\mu g/m^3$) and are within the range of human exposure to daily $PM_{2.5}$ concentrations commonly found (5-50 $\mu g/m^3$) in US cities, yet far below $PM_{2.5}$ levels (> 100 $\mu g/m^3$) regularly observed in India and China (Brook et al. 2010). The sensitivity of mice to $PM_{2.5}$ exposure is consistent with data from human studies showing that an acute increase in $PM_{2.5}$ of only 2.5 $\mu g/m^3$ (9.7±3.9 to 11.2±3.9 $\mu g/m^3$) enhances HOMA-IR (Brook et al. 2013b) and an increase of 10 $\mu g/m^3$ enhances the diabetes prevalence by 1% in chronically-exposed individuals living in the southeast US (Pearson et al. 2010).

Previous work with rodent models of diet-induced obesity has shown that early vascular insulin resistance could be a critical contributing factor to the subsequent development of systemic glucose intolerance and frank diabetes (Kim et al. 2008; Kubota et al. 2011). In these models the early decrease in the insulin-sensitivity in the aorta (after 1-2 week on HFD) is followed by the development of insulin resistance in the skeletal muscle and liver (8 weeks) and then in the adipose tissue (14 weeks) (Kim et al. 2008). Moreover, vascular insulin resistance

due to the deletion of endothelial-specific insulin-receptor substrate-2 (IRS-2) has been found to be sufficient for the development of systemic glucose intolerance (Kubota et al. 2011). In view of this data, we suggest that like in the development of diet-induced insulin resistance, the development of $PM_{2.5}$-induced insulin resistance also starts in the vasculature and then extends to other peripheral organs such as the skeletal muscle, the liver and the adipose tissue. Nevertheless, we cannot exclude the possibility that changes in the liver, skeletal muscle, or adipose tissue also contribute to the systemic effects of $PM_{2.5}$. Chronic exposure to PM induces hepatic ER stress (Laing et al. 2010) -- a process implicated in the development of insulin resistance and diabetes (Hotamisligil 2010), which could potentially contribute to the development of systemic insulin resistance induced by CAP exposure, especially in the setting of diet-induced obesity. Similarly, inflammation in the adipose tissue could be a contributing factor. Indeed we found that macrophage infiltration in the adipose tissue of HFD-fed mice was increased by CAP exposure (see Supplemental Material, Figure S1D). This could also contribute to the development of systemic insulin resistance, as previous studies have shown that diet-induced obesity is associated with macrophage infiltration in the adipose tissue and depletion of macrophages prevents systemic insulin resistance (Bu et al. 2013). We found that even though CAP exposure enhanced the mRNA levels of TNF-α and MIP-1α in the adipose tissue of HFD-fed mice, presumably due to an increase in infiltrating macrophages, it decreased HFD-induced increase in adipose tissue leptin mRNA levels and adipocyte hypertrophy (see Supplemental Material, Figure S1C-E). The mechanisms underlying these effects of $PM_{2.5}$ and their contribution to the development of systemic insulin resistance remain unclear, but excessive TNF-α production in the adipose tissue could induce lipolysis (Suganami and Ogawa 2010) and thereby decrease adipocyte size.

Although the mechanisms by which HFD induces insulin resistance remain unclear, it has been suggested that oxidative stress plays a critical role in this process (Paneni et al. 2015). Therefore, at least in principle, an increase in ROS production in $PM_{2.5}$ exposed mice (and humans) could be one mechanism by which $PM_{2.5}$ exposure increases insulin resistance, as also suggested by a previous study with p47(phox) deficient mice (Xu et al. 2010). A critical role of ROS in mediating the effects of $PM_{2.5}$ is supported by our observations that CAP exposure increases the levels of antioxidant enzymes such as ecSOD in the lungs and that $PM_{2.5}$-induced vascular insulin resistance is prevented by TEMPOL, which catalyzes the disproportionation of superoxide (Krishna et al. 1996). Even though it remains unclear whether the increased sensitivity of HFD mice to $PM_{2.5}$ is due to excessive perturbations in the tissue redox signaling, and inadequate antioxidant response, our current data indicate that vascular insulin resistance in $PM_{2.5}$-exposed mice may be attributable, at least in part, to oxidative stress induced by $PM_{2.5}$ exposure. $PM_{2.5}$ contains several pro-oxidant molecules, particularly metals such as iron, zinc, nickel and chromium that can trigger the formation of superoxide and other related ROS, and polycyclic aromatic hydrocarbons and quinones that can undergo redox cycling to generate ROS (Ghio et al. 2012). Indeed, compositional analysis of the Louisville CAP of our exposure studies showed high levels of iron (8-12%, see Supplemental Material, Table S2). Consistent with a central role of ROS in mediating PM toxicity, it has been shown that the toxicity of airborne particles correlates with their ability to generate ROS and that exposure to such particles induces oxidative stress in different tissues and cell lines (Ghio et al. 2012).

If the vascular effects of $PM_{2.5}$ are mediated by oxidative stress, then where is this stress initiated and how does this affect vascular insulin signaling? It has been suggested previously that systemic oxidative stress is due to either $PM_{2.5}$ particles deposited in the lung that induce pulmonary oxidative stress, which then spreads systemically, or that particles or particle

constituents leak from the lung into the systemic circulation and then cause oxidative injury in peripheral tissues (Brook et al. 2010). Our findings that overexpressing of ecSOD only in the lung prevents vascular insulin resistance suggest that pulmonary oxidative stress *per se* is sufficient for $PM_{2.5}$-induced vascular insulin resistance and that vascular insulin resistance could not be attributed to a diffusion of particles or particle constituents from the lung into the circulation but is an indirect consequence of pulmonary oxidative stress. Nevertheless, how oxidative stress is transmitted from the lung to the blood vessels remains unclear. Kampfrath and co-workers (Kampfrath et al. 2011) have suggested that pulmonary oxidative stress generates oxidized lipids (e.g., POVPC) that diffuse from the lung to peripheral tissues triggering tissue-specific injury. However, diffusible lipids that arise in the lung and interfere with vascular insulin signaling are yet to be identified unambiguously and further experiments are required to elucidate their role in inducing vascular insulin resistance secondary to pulmonary oxidative stress. Nonetheless, our current observations show that pulmonary oxidative stress per se plays a central role in peripheral effects of $PM_{2.5}$ and point to a fundamental, but poorly understood, link between pulmonary oxidative stress and vascular insulin signaling. Like $PM_{2.5}$ exposure, other insults that cause pulmonary oxidative stress such as exposure to tobacco smoke or ozone also have been associated with the development of systemic insulin resistance (Bass et al. 2013; Henkin et al. 1999; Vella et al. 2014). Similarly, pulmonary diseases such as microbial infections (Wang et al. 2009) and asthma (Gulcan et al. 2009) also are associated with insulin resistance and an increased risk for diabetes. Although it remains to be seen whether an increase in pulmonary oxidative stress induced by various toxicological or pathological insults is a general cause of insulin resistance and diabetes, it is tempting to speculate that the vascular endothelium is particularly sensitive to oxidants generated in the lung and that there is an

underappreciated link between pulmonary oxidative stress and the onset and precipitation of metabolic disease.

CONCLUSIONS

We found that upon exposure to $PM_{2.5}$ mice develop vascular insulin resistance, associated with vascular inflammation and dysfunction. These changes occurred even in the absence of HFD-feeding, but were similar to vascular changes seen in models of diet-induced obesity. The results of our experiments with mice treated with the antioxidant TEMPOL and in mice overexpressing ecSOD in the lung suggest that vascular insulin resistance is secondary to oxidative stress in the lung. On the basis of these observations, we suggest a model in which $PM_{2.5}$ deposited in the lung generates ROS, which in turn generate a diffusible mediator(s) that interferes with vascular insulin signaling by attenuating insulin-stimulated Akt and eNOS phosphorylation in blood vessels (see Supplemental Material, Figure S5). Further studies are required to identify the mediators of this process and to determine how secondary oxidative products arising in the lung cause vascular insulin resistance. Nonetheless, our observations suggest that the cardiovascular, and possibly the metabolic, effects of $PM_{2.5}$ could be mitigated by improving lung health or by targeting antioxidant interventions to the lung. Increasing the antioxidant capacity of the lung is likely not only to delay the progression of chronic cardiopulmonary injury due to chronic $PM_{2.5}$ exposure, but might also mitigate its acute cardiovascular effects. Conversely, conditions associated with a decrease in the antioxidant capacity of the lung, such as smoking, asthma, advanced age or influenza could increase the susceptibility of affected individuals to the cardiovascular effects of PM. Indeed some studies have suggested that smoker (Miller et al. 2007; Pope et al. 2004), individuals of advanced age (Andersen et al. 2008; Samoli et al. 2008), and individuals with asthma (Yeatts et al. 2007) or

influenza (Chen et al. 2011) are much more susceptible to cardiovascular effects of PM exposure, but more work is needed to establish whether lung health is a risk factor for the cardiovascular effects of PM exposure. Our current model provides a new template for understanding how $PM_{2.5}$ exposure, by inducing vascular insulin resistance, could simultaneously affect disparate cardiovascular processes such as thrombosis, autonomic dysfunction, blood pressure regulation, and atherogenesis as well as metabolic changes critical for the development of diabetes.

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Table 1: Systemic effects of CAP exposure for 30 days (Study I).

Parameter, unit, n	air	CAP	*p*	air+HFD	CAP+HFD	*p*
Body weight, g , 8	27.88±0.58	27.76±0.53	*0.882*	31.69±0.87	32.25±0.92	*0.665*
Glucose, mg/dL, 8	151±9	162±9	*0.374*	175±11	171±10	*0.781*
Insulin, ng/mL, 4-5	0.38±0.03	0.36±0.03	*0.660*	0.55±0.01	0.69±0.04	*0.019*
HOMA-IR, 4-5	4.2±0.3	3.9±0.4	*0.609*	6.4±0.3	8.6±0.6	*0.014*
HOMA-β (%), 4-5	42.5±3.7	44.9±7.4	*0.780*	56.5±4.6	66.2±12.4	*0.490*
Adiponectin, ng/mL, 5	13.38±3.01	11.32±1.55	*0.559*	11.34±1.56	10.06±2.87	*0.704*
Leptin, ng/mL, 5	0.39±0.03	0.35±0.04	*0.385*	0.77±0.21	0.78±0.22	*0.975*
Resistin, ng/mL, 5	3.01±0.56	3.12±0.58	*0.899*	3.24±0.28	3.28±0.40	*0.928*
TNF-α, pg/mL, 5	4.24±0.80	2.60±0.35	*0.108*	4.09±0.78	4.10±1.00	*0.991*
IL-6, pg/mL, 5	17.29±4.03	16.89±2.10	*0.933*	13.98±0.81	9.98±2.88	*0.230*
TG, mg/dL,6-8	19.44±0.73	19.11±3.36	*0.911*	17.50±2.29	21.52±3.19	*0.323*
Cholesterol, mg/dL, 6-8	79.56±2.42	113.09±5.53	*0.001*	129.96±3.76	154.69±8.74	*0.021*
HDL, mg/dL, 6-8	57.40±2.07	65.70±5.93	*0.187*	95.14±5.27	108.02±5.60	*0.116*
LDL, mg/dL, 6-8	10.38±0.58	14.14±1.53	*0.024*	19.79±2.36	17.36±1.03	*0.360*
HDL:LDL, 6-8	5.36±0.11	4.73±0.24	*0.030*	5.05±0.35	6.34±0.44	*0.038*
TP, g/dL, 5-8	5.22±0.13	5.32±0.17	*0.653*	5.47±0.19	5.64±0.21	*0.544*
Albumin, g/dL, 5-8	3.62±0.15	3.56±0.14	*0.765*	3.36±0.07	3.70±0.16	*0.068*
Albumin :TP, 5-8	0.69±0.02	0.67±0.01	*0.276*	0.62±0.02	0.66±0.02	*0.115*
ALT, U/L, 5-8	30.60±5.70	30.91±2.98	*0.962*	30.03±3.35	27.84±3.21	*0.644*
AST, U/L, 4-8	48.39±6.71	62.33±13.10	*0.380*	72.29±11.52	72.48±4.96	*0.988*
CK, U/L, 4-8	141.22±73.49	171.53±83.83	*0.797*	245.98±43.10	241.99±25.24	*0.937*

Blood and plasma parameter were measured in control or HFD-fed mice exposed for 30 days to air or CAP. Data are mean ± SE; p, air vs. CAP. Abbr.: HOMA-IR (=fasting blood glucose [mmol/L] x fasting plasma insulin levels [mU/L]/22.5); HOMA-β (=20 x fasting plasma insulin levels [mU/L]/ fasting blood glucose [mmol/L]-3.5), %; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatinine kinase; TG, Triglycerides; TP, Total Protein; HDL, high-density lipoprotein cholesterol; IL-6, interleukin 6; LDL, low-density lipoprotein cholesterol; TNF-α, tumor necrosis factor α..

Table 2: Systemic effects of CAP exposure for 9 days (Study III).

Parameter, unit, n	air	CAP	*p*	air+HFD	CAP+HFD	*p*
Body weight, g, 14	27.43±0.46	28.52±0.61	*0.163*	29.36±0.48	30.67±0.40	*0.046*
Glucose, mg/dL, 14	160±9	156±7	*0.700*	167±9	160±6	*0.489*
Insulin, ng/mL, 4	0.46±0.07	0.47±0.06	*0.898*	0.85±0.12	0.77±0.13	*0.653*
HOMA-IR, 4	4.9±1.0	4.5±0.5	*0.689*	8.4±0.5	8.5±1.6	*0.949*
HOMA-β (%), 4	42.6±4.8	48.4±8.5	*0.578*	62.6±6.0	73.1±11.0	*0.434*
Adiponectin, ng/mL, 4	9.79±1.90	7.02±0.14	*0.197*	11.16±2.62	13.17±2.62	*0.600*
Leptin, ng/mL, 4	0.44±0.05	0.50±0.09	*0.586*	0.98±0.33	0.84±0.18	*0.727*
Resistin, ng/mL, 4	1.90±0.87	1.92±0.41	*0.983*	2.96±1.20	2.33±0.51	*0.690*
TNF-α, pg/mL, 4	4.53±0.44	4.80±1.33	*0.851*	4.11±0.64	2.94±0.47	*0.212*
IL-6, pg/mL, 4	8.41±2.45	4.28±1.25	*0.184*	8.45±1.25	8.45±1.13	*0.999*
TG, mg/dL, 9-10	33.56±5.13	37.69±4.96	*0.571*	16.80±1.79	18.29±2.51	*0.634*
Cholesterol, mg/dL, 9-10	66.07±3.79	75.94±6.55	*0.222*	110.41±3.62	121.60±6.19	*0.136*
HDL, mg/dL, 9-10	45.44±1.96	50.81±3.08	*0.170*	82.56±2.51	93.70±6.53	*0.128*
LDL, mg/dL, 9-10	10.38±0.44	12.54±1.11	*0.100*	18.62±1.35	16.69±0.61	*0.210*
HDL:LDL, 9-10	4.42±0.21	4.16±0.18	*0.358*	4.62±0.31	5.78±0.32	*0.019*
TP, g/dL, 9-10	4.32±0.12	4.58±0.15	*0.198*	4.33±0.11	4.39±0.08	*0.670*
Albumin, g/dL, 9-10	3.13±0.07	3.25±0.09	*0.297*	2.88±0.06	2.93±0.05	*0.511*
Albumin :TP,9-10	0.73±0.01	0.71±0.01	*0.242*	0.67±0.01	0.67±0.01	*0.818*
ALT, U/L, 9-10	19.98±1.00	26.36±3.16	*0.084*	20.14±1.94	17.59±1.01	*0.260*
AST, U/L, 9-10	42.02±4.11	51.08±4.13	*0.140*	54.78±2.96	53.99±3.25	*0.856*
CK, U/L, 8-10	135.58±16.99	132.64±18.01	*0.909*	184.11±29.64	183.60±28.47	*0.991*

Blood and plasma parameter were measured in control or HFD-fed mice exposed for 9 days to air or CAP. Data are mean ± SE; p, air vs. CAP. Abbr.: HOMA-IR (=fasting blood glucose [mmol/L] x fasting plasma insulin levels [mU/L]/22.5); HOMA-β (=20 x fasting plasma insulin levels [mU/L]/ fasting blood glucose [mmol/L]-3.5), %; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatinine kinase; TG, Triglycerides; TP, Total Protein; HDL, high-density lipoprotein cholesterol; IL-6, interleukin 6; LDL, low-density lipoprotein cholesterol; TNF-α, tumor necrosis factor α.

FIGURE LEGENDS

Fig. 1: **Effects of CAP exposure on systemic glucose homeostasis.** Mice maintained on control diet (13% kcal fat) or placed on a high-fat diet (HFD, 60% kcal fat) were exposed for 30 days to air or CAP (Study I). After 25 days of exposure, systemic glucose tolerance was tested in both control diet (A) and HFD-fed (B) mice. The total excursion of glucose in the blood was calculated by integrating the area under the curve (AUC, inset). Data are mean ± SE ($^{\#}$ $p < 0.05$ air vs. CAP; n = 8).

Fig. 2: **Effects of CAP exposure on the organ-specific insulin sensitivity.** Western blot analysis of Akt phosphorylation in lung (A) and heart (B) and phosphorylation of Akt (i) and eNOS (ii) in aorta (C) and skeletal muscle (D) in mice injected with saline or insulin (1.5 U/kg). Mice fed control diet (10 % kcal fat) or placed on high-fat diet (HFD, 60 % kcal fat) were exposed for 30 day to air or CAP (Study II). Data are mean ± SE normalized to controls (* $p < 0.05$ saline vs. insulin; NS, not significant; $^{\#}$ $p < 0.05$, $^{+}$ $p < 0.1$ air vs. CAP; n = 4).

Fig. 3: **CAP exposure induces aortic insulin resistance and vascular inflammation.** (A) Western blot analysis of the insulin-stimulated (100 nM, 15min) phosphorylation of Akt (i), eNOS (ii) and ERK (iii) and the abundance of IκBα (iv) in aortas isolated from mice maintained on control diet (13 % kcal fat) or placed on a high-fat diet (HFD, 60 % kcal fat) exposed for 9 days to air or CAP (Study III). The continuous Western blot to detect the abundance of IκBα is shown in the Supplemental Material, Figure S4A. Data are mean ± SE normalized to controls ($^{\#}$ $p < 0.05$ vs. air-exposed control diet-fed mice; phospho-Akt, n = 8; phospho-eNOS, n = 5; phospho-ERK, n = 6; IκBα, n = 4). (B) Dose dependency of CAP-induced

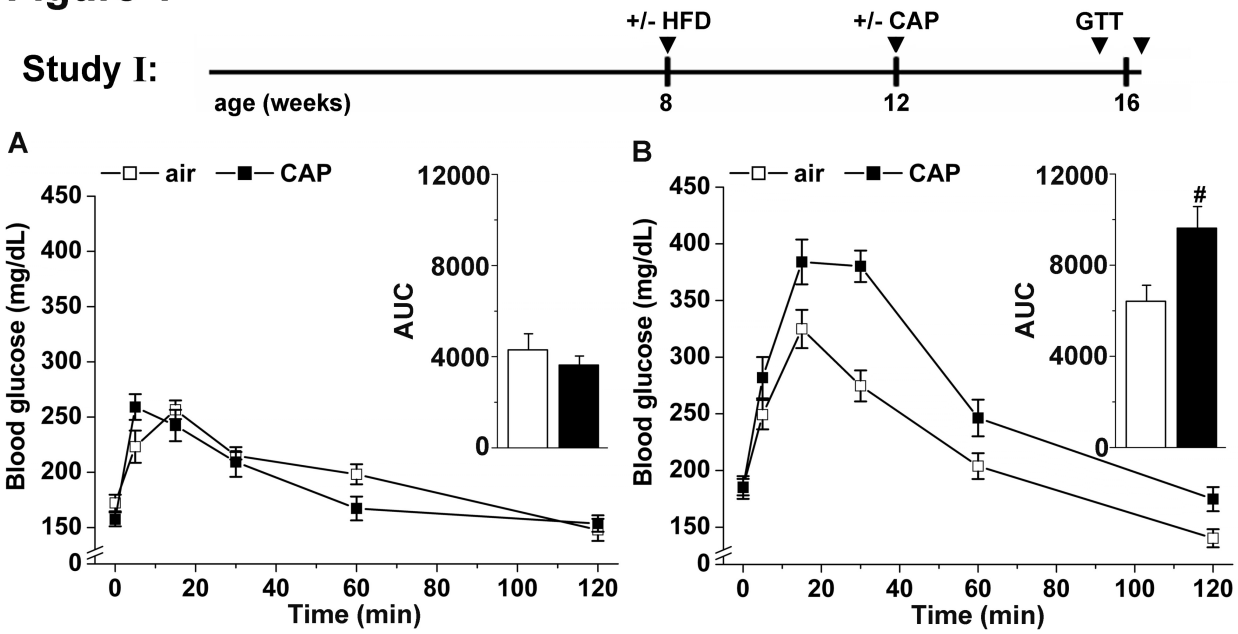
vascular insulin resistance was analyzed in insulin-stimulated aortas isolated from mice exposed for 9 or 30 days to air or CAP. For each exposure performed between 2010 and 2013 (see Supplemental Material, Table S4) the extent of insulin-induced Akt phosphorylation in the aorta was measured by Western analysis as described. Data are shown as discrete points of insulin-induced Akt phosphorylation (mean ± SE, in % of air-exposed controls) and the cumulative CAP dose for each exposure, and the curve is a best fit of a first-order exponential equation [($y=y_0 + a_1 \exp(-x/t_1)$] to the data. (C) Western blots analysis of the plasmatic abundance of protein-acrolein adducts (loading controls are shown in the Supplemental Material, Figure S4C) and plasma TBARS levels (C, inset) in mice exposed for 9 days to air or CAP. Data are mean ± SE ($^{\#}$ $p < 0.05$, $^{+}$ $p < 0.1$ air vs. CAP; n = 5).

Fig. 4: **TEMPOL treatment prevents CAP-induced vascular insulin resistance and inflammation.** Western blot analysis of (A) the insulin-stimulated phosphorylation of Akt and (B) abundance of IκBα in the aorta of mice treated with water or TEMPOL (1mM, in drinking water) exposed to air or CAP for 9 days (Study IV). The continuous IκBα Western blot is shown in the Supplemental Material, Figure S4B. Data are mean ± SE normalized to controls (* $p < 0.05$ control vs. insulin; $^{\#}$ $p < 0.05$ air vs. CAP; phospho-Akt, n = 5-10; IκBα, n = 4-5).

Fig. 5: **CAP-induced vascular insulin resistance and inflammation are prevented in lung-specific ecSOD transgene (ecSOD-Tg) mice.** (A) Western blot analysis of the pulmonary ecSOD protein abundance (n =4) in WT mice exposed to air or CAP for 9 days. **(**B) Western blots of the transgene ecSOD (*t*-ecSOD) protein abundance in lung and aorta isolated from WT mice exposed to air or CAP for 9 days and

ecSOD-Tg mice (Study V). Western blot analysis of the (**C**) pulmonary abundance of protein-acrolein adducts (loading controls are shown in the Supplemental Material, Figure S4D, n = 4-5), (D) aortic insulin-stimulated Akt phosphorylation (n = 5-8) and (E) aortic IκBα abundance (n = 8-12) in WT and ecSOD-Tg mice exposed for 9 days to air or CAP. Data are mean ± SE normalized to controls (* $p < 0.05$ control vs. insulin; $^{\#}$ $p < 0.05$, $^{+}$ $p < 0.1$ air vs. CAP).

Figure 1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+/- HFD
+/- CAP
GTT
age (weeks)
8
12
16
A
air
CAP
Blood glucose (mg/dL)
Time (min)
AUC
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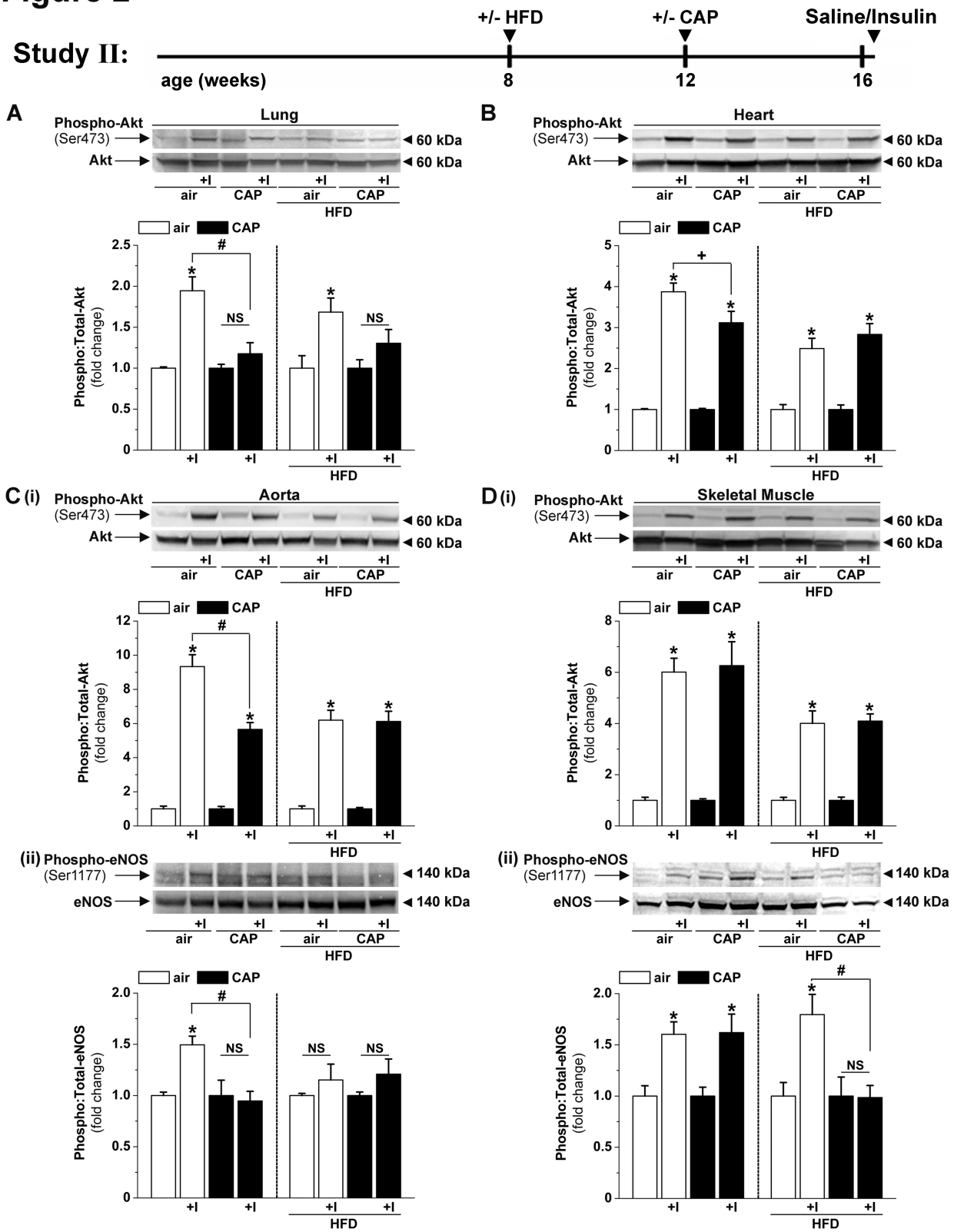
air
CAP
Blood glucose (mg/dL)
Time (min)
AUC
#

Figure 2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+/- CAP
Saline/Insulin
age (weeks)
8
12
16
A
Lung
B
Heart
C (i)
Aorta
D (i)
Skeletal Muscle
Phospho-Akt
(Ser473)
Akt
60 kDa
air
CAP
HFD
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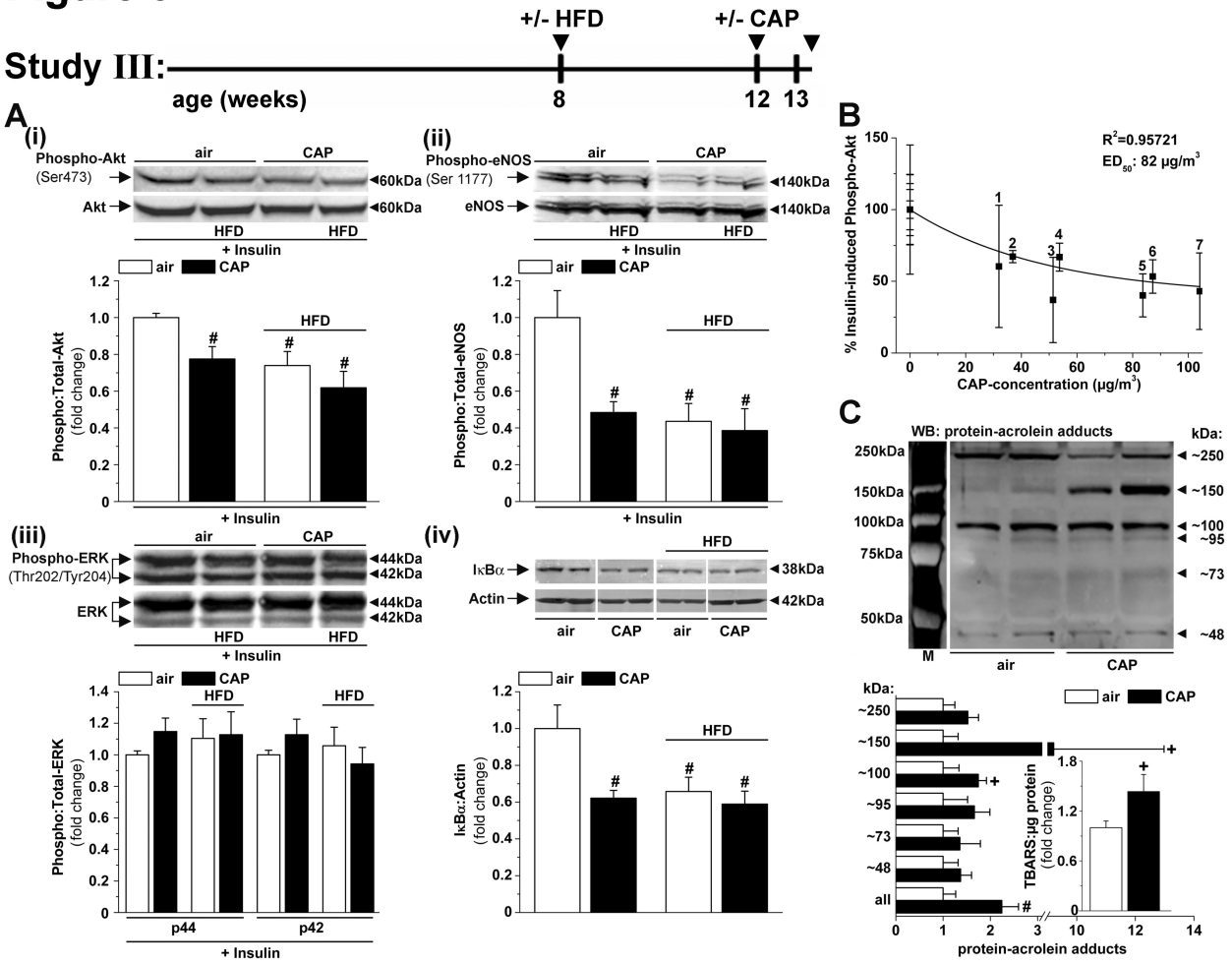
140 kDa
Phospho:Total-eNOS

Figure 3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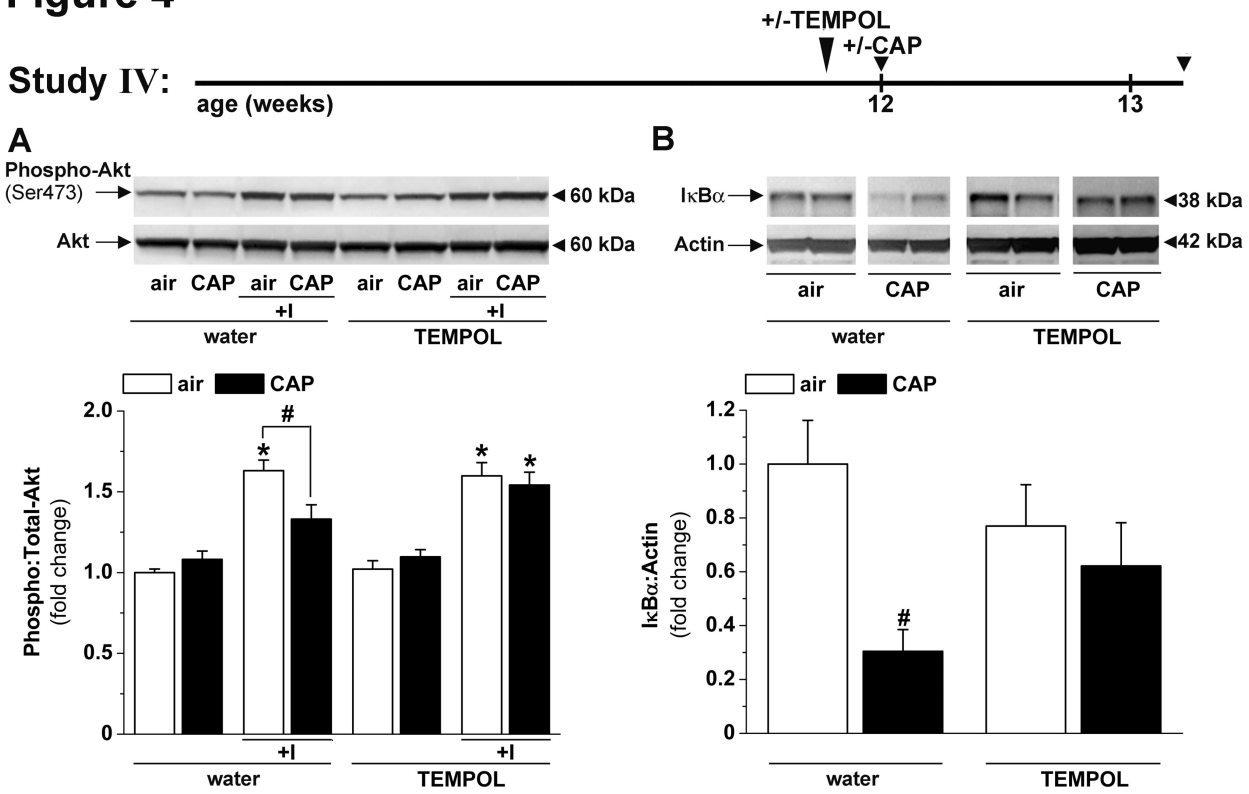
+/- CAP
age (weeks)
8
12
13
A
(i)
Phospho-Akt (Ser473)
Akt
60kDa
air
CAP
HFD
+ Insulin
Phospho:Total-Akt (fold change)
(ii)
Phospho-eNOS (Ser 1177)
eNOS
140kDa
Phospho:Total-eNOS (fold change)
(iii)
Phospho-ERK (Thr202/Tyr204)
ERK
44kDa
42kDa
Phospho:Total-ERK (fold change)
p44
p42
(iv)
IκBα
Actin
38kDa
IκBα:Actin (fold change)
B
R²=0.95721
ED50: 82 µg/m³
% Insulin-induced Phospho-Akt
CAP-concentration (µg/m³)
C
WB: protein-acrolein adducts
kDa:
250kDa
150kDa
100kDa
75kDa
50kDa
M
~250
~150
~100
~95
~73
~48
TBARS:µg protein (fold change)
all
protein-acrolein adducts

Figure 4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+/-CAP
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13
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Phospho-Akt
(Ser473)
Akt
60 kDa
60 kDa
air CAP air CAP air CAP air CAP
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water
TEMPOL
air
CAP
Phospho:Total-Akt
(fold change)
2.0
1.5
1.0
0.5
0
#
*
*
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+I
+I
water
TEMPOL
B
IκBα
Actin
38 kDa
42 kDa
air
CAP
air
CAP
water
TEMPOL
air
CAP
IκBα:Actin
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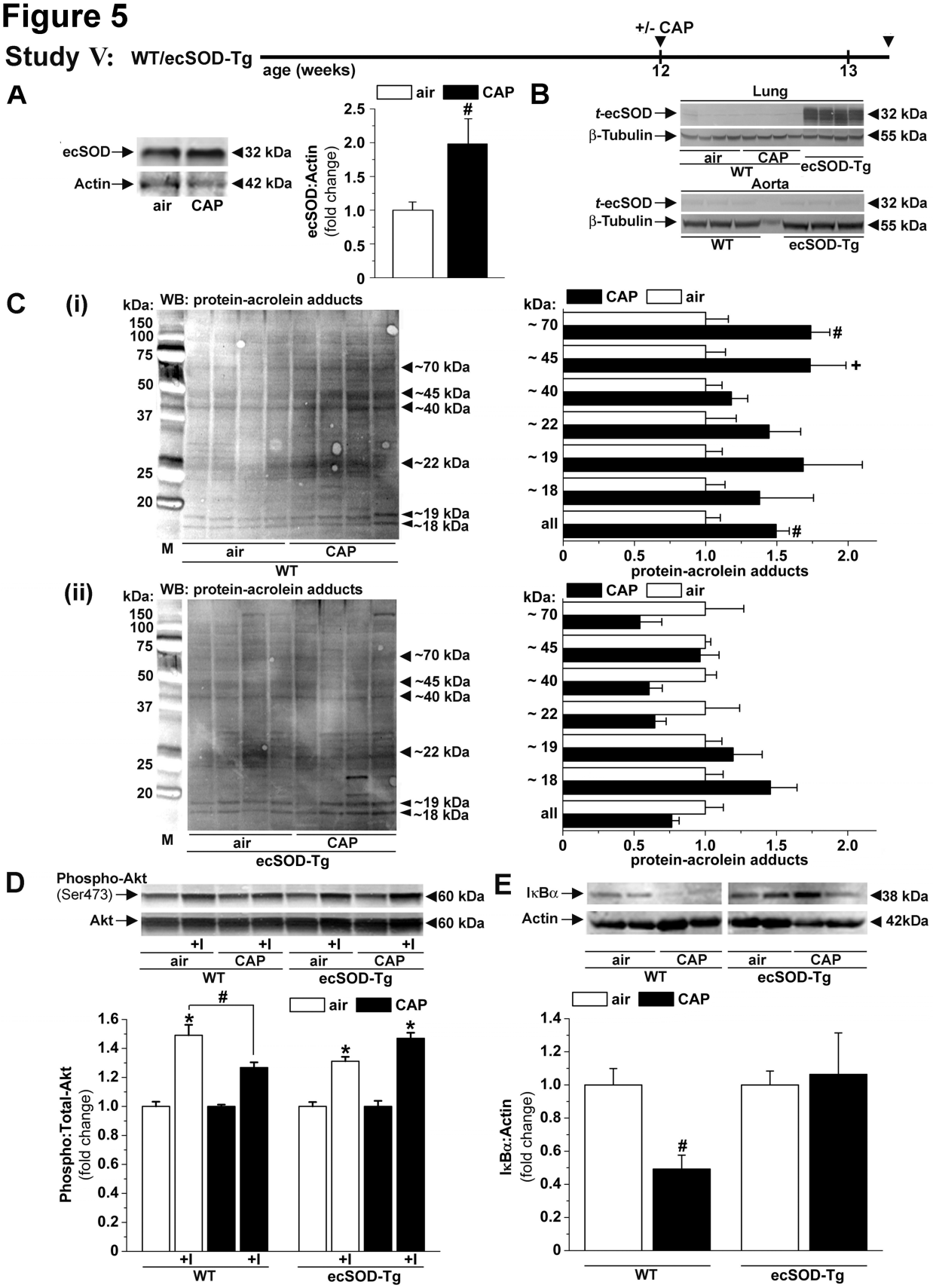
1.2
1.0
0.8
0.6
0.4
0.2
0
#
water
TEMPOL

Figure 5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age (weeks)
+/- CAP
12
13
A
ecSOD
Actin
32 kDa
42 kDa
air
CAP
ecSOD:Actin (fold change)
B
Lung
t-ecSOD
β-Tubulin
55 kDa
WT
ecSOD-Tg
Aorta
C
(i)
WB: protein-acrolein adducts
kDa:
~70 kDa
~45 kDa
~40 kDa
~22 kDa
~19 kDa
~18 kDa
M
protein-acrolein adducts
(ii)
D
Phospho-Akt (Ser473)
Akt
60 kDa
+I
Phospho:Total-Akt (fold change)
E
IκBα
38 kDa
Actin
42kDa
IκBα:Actin (fold change)